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Interaction between Core protein of classical swine fever virus with cellular IQGAP1 protein appears essential for virulence in swine

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ABSTRACT

Here we show that IQGAP1, a cellular protein that plays a pivotal role as a regulator of the cytoskeleton interacts with Classical Swine Fever Virus (CSFV) Core protein. Sequence analyses identified residues within CSFV Core protein (designated as areas I, II, III and IV) that maintain homology to regions within the matrix protein of Moloney Murine Leukemia Virus (MMLV) that mediate binding to IQGAP1 [EMBO J, 2006 25:2155]. Alanine-substitution within Core regions I, II, III and IV identified residues that specifically mediate the Core-IQGAP1 interaction. Recombinant CSFV viruses harboring alanine substitutions at residues ^{207}ATI^{209} (I), ^{210}VVE^{212} (II), ^{213}GVK^{215} (III), or ^{232}GLYHN^{236} (IV) have defective growth in primary swine macrophage cultures. *In vivo*, substitutions of residues in areas I and III yielded viruses that were completely attenuated in swine. These data shows that the interaction of Core with an integral component of cytoskeletal regulation plays a role in the CSFV cycle.

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Introduction

Classical swine fever virus (CSFV) is a small, enveloped virus with a positive, single-stranded RNA genome that causes classical swine fever (CSF), a highly contagious disease of swine. CSFV, along with Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV), are members of the genus *Pestivirus* within the family *Flaviviridae* (Fauquet et al., 2005). The CSFV genome is approximately 12.3 kb and contains a single open reading frame encoding a polyprotein of 3898-amino-acids. Co- and post-translational processing of the polyprotein by cellular and viral proteases ultimately yields 11 to 12 final cleavage products (NH_2-Npro-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Rice, 1996). Structural components of the CSFV virion include the glycoproteins E^{rns}, E1, E2, and a nucleocapsid of unknown symmetry, the Core protein.

Within the *Flaviviridae* family, Core is encoded as the second product of the polyprotein. The Core protein of pestiviruses is a small, highly basic polypeptide that is cleaved at its N-terminus by Npro, an event critical for infectious particle production. The Core protein of BVDV has additionally been characterized as lacking significant secondary structures (Murray et al., 2008). It is known that the C-terminal of Core is cleaved by signal peptide peptidase. (Heimann et al., 2006; Rümenapf et al., 1998; Meyers et al., 1989), and it has been suggested that the CSFV Core protein influences regulation of cellular transcription (Liu et al., 1998) and also interacts with host SUMOylation proteins (Gladue et al., 2010).

Analysis of the Core protein of Hepatitis C Virus (HCV), another member of the *Flaviviridae* family, provides further insight into the possible functions of the Core protein of CSFV. HCV Core self-assembles into nucleocapsid-like particles in the presence of nucleic acids (Kunkel et al., 2001) and can directly interact with HCV RNA (Fan et al., 1999; Shimoike et al., 1999; Tanaka et al., 2000). Core can bind other HCV proteins such as NS5A and E1 (Goh et al., 2001; Lo et al., 1996; Masaki et al., 2008) and interacts with host cellular proteins (Jin et al., 2000; Mamiya and Worman., 1999; Otsuka et al., 2000; Yoshida et al., 2002; You et al., 1999), influencing HCV pathogenesis by modulation of signaling pathways, cell transformation and proliferation, regulation of cellular and viral gene expression, apoptosis, and alteration of lipid metabolism. (Giannini and Brechot, 2003; Levrero, 2006; Tellinghuisen and Rice, 2002; Lai and Ware., 2000; McLauchlan, 2000; Ray and Ray, 2001). HCV Core protein is capable of impairing the host's immune response, by interacting with cell molecules that results in suppression of IL-12 synthesis in human macrophages (Eisen-Vandervelde et al., 2004), T cell dysfunction (Yao

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et al., 2007), and inhibition of T-lymphocyte activation and proliferation (Chen et al., 1994; Kittlesen et al., 2000; Yao et al., 2001).

Although the role of CSFV structural glycoproteins in virus virulence has been studied in detail (Meyers et al., 1999; Risatti et al., 2005a, 2005b, 2006, 2007a, 2007b; van Rijn et al., 1994; van Gennip et al., 2004), knowledge about the role of Core protein on the outcome of CSFV infection in swine is limited. Recently, we have shown that CSFV Core protein interacts with proteins of the cellular SUMOylation pathway, SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme (Gladue et al., 2010). Substitution of Core residues in CSFV involved with binding to SUMO1 and UBC9 resulted in virus attenuation in swine. To further elucidate the role of Core protein in CSFV virulence, we expanded this previous study to identify additional host factors interacting with the Core protein during virus infection. Here we report that the cellular IQGAP1 protein interacts specifically with the CSFV Core protein. IQGAP1 is involved in a diverse set of protein–protein interactions and is a prominent regulator of the cytoskeleton (for a review, see Noritake et al., 2005; Brandt and Grosse, 2007). This protein binds monomeric G proteins: Cdc42 and Rac, likely mediating their effects in reorganizing the cytoskeleton (Fukata et al., 2002; Watanabe et al., 2004).

Specific interaction of IQGAP1 with the Moloney murine leukemia virus (MMLV) matrix (M) protein suggests its involvement in intracellular trafficking of the virus, an interaction that is essential for virus replication (Leung et al., 2006). Mutational studies performed in that report defined residues of MMLV M protein, critical for its interaction with IQGAP1. Based on sites within the MMLV M protein that recognize IQGAP1, four areas (I–IV) within the CSFV Core protein that potentially recognize IQGAP1 were identified. Substitution of Core native amino acid residues with alanine in areas I and III completely abolished the Core–IQGAP1 protein–protein binding whereas substitutions in areas II and IV only partially affected the interaction. CSFV mutants harboring substitutions in these four areas were developed to assess the importance of the Core–IQGAP1 protein interaction for virulence in swine. Remarkably, substitutions in areas I and III of the Core protein, significantly affecting IQGAP1 recognition, correlate with complete absence of virulence in swine. Therefore, the ability of CSFV Core protein to bind cellular IQGAP1 protein during infection plays a critical role in virus virulence within the swine host.

Results

CSFV structural Core protein binds swine IQGAP1 protein

To identify host cellular proteins that interact with CSFV Core protein, we constructed an N-terminal fusion of the Gal4 protein binding domain to the Core protein as 'bait' for the yeast two-hybrid system. Approximately 1×10^7 independent yeast colonies derived from a swine primary macrophage cDNA library containing 3×10^6 independent clones were screened. These colonies were selected for growth using -Leu/-Trp/-His/-Ade media. Plasmids were isolated from positive colonies and sequenced. In-frame proteins were retested for specificity to the Core protein. As a negative control proteins were tested for binding the Lam-BD protein. Several proteins were identified as specific binding partners for the CSFV Core protein (data not shown). One of these proteins, IQGAP1, was selected for further study since IQGAP1 functions as a prominent regulator of the cytoskeleton which likely plays a role in viral pathogenesis. IQGAP1 specifically bound Core protein when compared to binding of Lam-BD protein (Fig. 1).

Mapping areas of the CSFV Core protein critical for IQGAP1 recognition

Previous studies have described the binding of Moloney murine leukemia virus (MMLV) matrix (M) protein with IQGAP1 protein (Leung et al., 2006). Mutational studies defined residues of the MMLV M protein critical for interaction with IQGAP1, revealing a correlation between binding and virus replication. Based on the sites of MMLV M protein that recognize IQGAP1 (Leung et al., 2006), four (I–IV) homologous areas (Fig. 2) were identified in CSFV Core protein using the ClustalW software program (Thompson et al., 1994). CSFV mutants harboring alanine substitutions in place of native amino acid residues within these four areas were developed to assess whether these regions are important for IQGAP1 binding (Figs. 1 and 2). We produced the following mutant proteins containing Ala substitutions within the Core protein: CoreΔIQ.I (^{207}ATI^{209}), CoreΔIQ.II (^{210}VVE^{212}), CoreΔIQ.III (^{213}GVK^{215}), and CoreΔIQ.IV (^{232}GLYHN^{236}) (Table 1). These CoreΔIQ proteins were tested in the yeast two-hybrid system against the swine IQGAP1 protein. Interestingly, substitutions within areas I and III resulted in loss of ability to bind IQGAP1 (Fig. 1). In contrast, substitutions in areas II and IV only caused a decrease in the ability of the Core protein to bind the swine IQGAP1 protein. All CoreΔIQ mutated proteins maintained their ability to bind swine clathrin in the yeast two-hybrid at similar levels (data not shown), indicating that these mutated areas within the CoreΔIQ mutated proteins are areas specific for IQGAP1 binding.

Sequence analysis of the 100 amino acid residues of Core protein from geographically and temporally different CSFV isolates revealed a high degree of sequence similarity and conservancy at putative IQGAP1 target sites (Fig. 2), suggesting these sites play a critical role in the biology of CSFV. Additionally, the areas predicted in CSFV to bind IQGAP1 are highly conserved in BVDV and BDV, further suggesting a critical role for these residues in other pestiviruses (data not shown).

Replication of CoreΔIQ mutant viruses in vitro

To further evaluate the role of CSFV Core IQGAP1 binding sites in the biology of the virus, recombinant viruses based on virulent strain Brescia (BICv) were constructed, containing alanine substitutions in the previously described four critical IQGAP1 binding sites of the Core protein. Mutant viruses referred to as CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, and CoreΔIQ.IVv represent each of the four putative IQGAP1 binding sites within the CSFV Core protein (Table 1 and Fig. 2). Viruses were rescued from transfected cells by 4 dpi (days post-infection). Nucleotide sequences of viable rescued virus genomes were identical to parental DNA plasmids, confirming that only mutations at predicted mutated sites were reflected in rescued viruses.

In vitro growth characteristics of these mutant viruses were evaluated relative to parental BICv in a single-step growth curve. Primary swine macrophage cell cultures were infected at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero), and samples were collected at 72 h post-infection (hpi). All mutant viruses exhibited about one log10 decrease in titer when compared with parental BICv (Fig. 3), suggesting that all mutant viruses have an *in vitro* growth defect when compared to parental BICv.

Effect of Core–IQGAP interactions on the cell cytoskeleton

The effects of viral infection on γ-tubulin and vimentin were examined to compare the distribution of microtubules and intermediate filaments in cells infected with BICv, CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, CoreΔIQ.IVv, to that seen in uninfected cells (Fig. 5). In uninfected cells, the microtubules (visualized with antibodies recognizing γ-tubulin) as well as the intermediate filaments (visualized with antibodies recognizing vimentin), were arranged in a filamentous network running throughout the cytoplasm. In BICv-infected cells, γ-tubulin was rearranged into a ring surrounding the nucleus. When vimentin was examined a similar rearrangement was observed in BICv infected cells. Examining the appearance of γ-tubulin and vimentin in infected cells also revealed differences between the effects of BICv and CoreIQ viruses. Infection with CoreΔIQ.IIv and CoreΔIQ.IVv led to a rearrangement of both markers, γ-tubulin and vimentin, into a ring

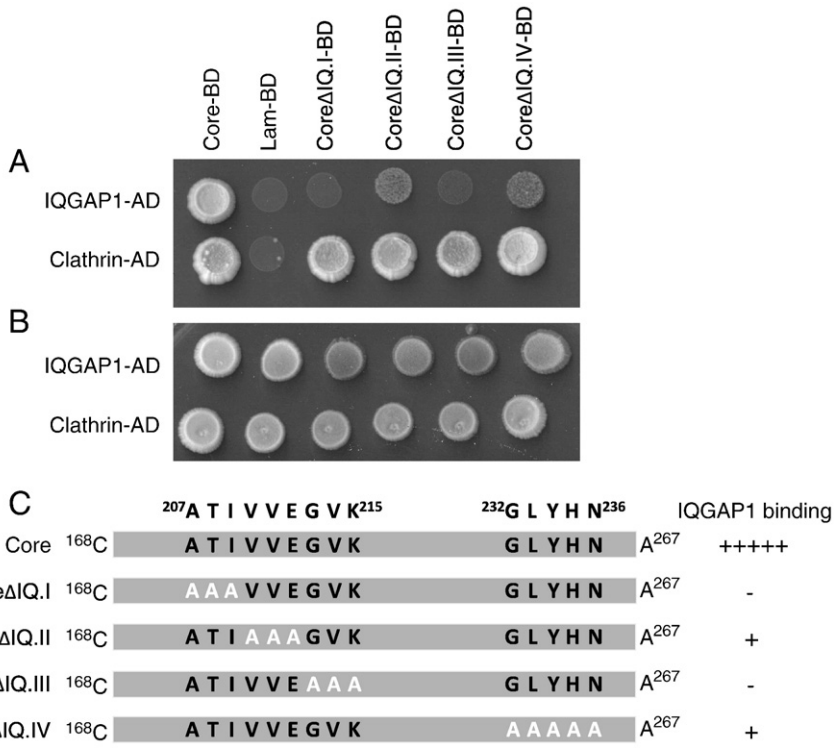

Fig. 1. Reactivity of CSFV wild-type and mutant Core proteins with IQGAP1 protein or Clathrin protein (positive control) in the yeast two-hybrid system. Wild-type Core and mutants I, II, III and IV proteins were tested for their ability to bind IQGAP1 protein. Yeast growth on selective SD-Ade-His-Leu-Trp media (A), and growth on non-selective SD-Leu-Trp media (B). Schematic representation of putative IQGAP1 binding motifs found in CSFV Core protein (C).

surrounding the nucleus similar to the one observed with BICv. In contrast, when CoreΔIQ.Iv and CoreΔIQ.IIIv were studied, both proteins, γ-tubulin and vimentin, retained a radial pattern as seen in uninfected cells (Fig. 5).

Evaluation of the role of CSFV Core IQGAP1 binding sites in CSFV virulence in swine

To examine the *in vitro* effects of deletion on Core protein interaction with IQGAP1 protein, all four CoreΔIQv mutants were intranasally (IN) inoculated into naïve swine, at doses of 10^5 $TCID_{50}$. After inoculation, survival of pigs inoculated with mutant viruses was assessed relative to lethal exposure of control animals infected with virulent BICv. The swine were monitored daily for clinical disease. BICv exhibited a characteristic virulent phenotype (Table 2); none of the control pigs survived the infection, dying or being euthanized around 8 dpi. Interestingly, viruses CoreΔIQ.Iv and CoreΔIQ.IIIv were completely attenuated in swine. Animals survived the infection and remained clinically normal throughout the observation period (21 days) with only one animal out of five infected with CoreΔIQ.IIIv presenting a transient rise in body

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BICv AY578687        CSDDGASASKEKKPDRINKGKLKIAPKEHEKDSRTKPPDATIVVEGVKYQVKKKGKVKGKNTQDGLYHNKNKPPESRKKLEKALLAWAVIAIMLYQPVAA
                                                             I  II III                  IV
Alfort 187 X87939    .......G.....................................................................................E.
LOM EU789580         .......G.....................................................................................E.
cF114 AF333000       .......G.....................................................................................E.
Alfort A19 U90951    .......G.....................................................................................E.
GPE- D49533          .......G.....................................................................................E.
Shimen AY775178      .......G.....................................................................................T.......E.
JL1(06)EU497410      .......G.....................................................................................T.......E.
SWH DQ127910         .......G.....................................................................................T.......E.
Thiverval EU490425   .......G.....................................................R.....................T.............E.
Eystrup NC002657     .......G.....................................................S.....................................E.
ALD D49532           .......G.....................................................S.....................................E.
CAP X96550           .......G.....................................................R.....................V.....E.
Glentorf U45478      .......G.....................................................R.....................V.....E.
HCLV AF531433        .......G.....................R...............................................................E.
Strain C AY805221    .......G.....................R...............................................................E.
Riems AY259122       .......G.....................R...............................................................E.
CS AY578688          .......G.............T.....................................................................
GXWZ02 AY367767      ......GG..D.....M.............K.....................................................VT.V.......
virus 39 AF407339    .......G..D.....M.............K..S.....................R.....................V..V.......
Spain01 FJ265020     .......GN.D.....M.............K.....................I.....................................V.......
96TD AY554397        ....S.GG..D.....MS............K.....................N.V.....................T.V.......
0406Taiwan AY568569  ......GG..D.....M.............K.....................................K.....V.....V.
Paderborn AY072924   ......GG.E.....M.............K.....................................T.V.......
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Fig. 2. Multiple alignments of CSFV Core proteins revealed the presence of highly conserved putative IQGAP1 sites I, II, III, and IV (bold underlined). Shaded are Lys residues involved with Core-SUMO-1 and Core-UBC9 protein–protein interactions as described by Gladue et al. (2010). Shown here is a comparison between geographically and temporally separated viruses.

Table 1
Set of CSFV CoreΔIQ mutant viruses constructed in this study.

Core wild type sequence	[a]Core mutant sequence	Mutant virus
^{207}ATI^{209}	^{207}AAA^{209}	CoreΔIQ.Iv
^{210}VVE^{212}	^{210}AAA^{212}	CoreΔIQ.IIv
^{213}GVK^{215}	^{213}AAA^{215}	CoreΔIQ.IIIv
^{232}GLYHN^{236}	^{232}AAAAA^{236}	CoreΔIQ.IVv

[a] Amino acid position relative to CSFV BICv polyprotein.

temperature. In contrast, animals infected with mutant CoreΔIQ.IIv or CoreΔIQ.IVv, although presenting less severe CSF symptoms than those infected with BICv, died around 12 and 14 dpi, respectively (Table 2). Therefore, there appears to be a close correlation between disrupting Core–IQGAP1 protein–protein binding as detected in the yeast two-hybrid system and the induction of virus attenuation.

Virus shedding (detected in tonsil scrapings and nasal swabs) and viremia in CoreΔIQ.Iv and CoreΔIQ.IIIv-inoculated animals were almost undetectable (sensitivity of detection ≥ 1.8 $TCID_{50}$/ml) at all time points sampled (Fig. 4). Interestingly, high virus loads in blood, nasal swabs, and tonsil scrapings were observed in animals infected with CoreΔIQ.IIv or CoreΔIQ.IVv although those titers were detected at a later time during the infection when compared to BICv-infected pigs (Fig. 4). Similarly, a delayed onset of disease was observed in animals inoculated with these mutant viruses relative to wild-type infected animals.

Animals that survive inoculation with CoreΔIQ.Iv and CoreΔIQ.IIIv when challenged with virulent BICv by 28 dpi were not protected, succumbing to the infection as naïve pigs (data not shown). Antibodies against E2 and E^{rns} were not detected in these animals by the time of challenge as measured by ELISA tests (CSF SERO ELISA and CHEKIT-CSF-MARKER Kits, Idexx Laboratories, Westbrook, ME, USA).

Discussion

Here we describe a specific interaction between IQGAP1 protein, a major cytoskeleton regulator, with CSFV Core protein. Substitution of residues ^{207}ATI^{209} or ^{213}GVK^{215} within Core, disrupting Core–IQGAP1 protein–protein interaction completely, correlates with the induction of virus attenuation *in vivo*. Partial disruption of Core–IQGAP1 binding by substituting Core residues ^{210}VVE^{212} or ^{232}GLYHN^{236} resulted in viruses inducing a delayed onset of CSF, suggesting that the degree of attenuation closely correlates with the ability of the Core protein to interact with IQGAP1, and that this swine–host protein interaction plays an important role in CSFV pathogenesis.

CoreΔIQ viruses showed a slight growth defect in cultured primary swine macrophages. Impaired replication observed *in vitro* usually correlates with virus attenuation *in vivo*, with animals overcoming the infection, likely because of poor virus replication *in vivo* and a rapid clearance of mutant viruses mediated by the host immune response. In fact, CoreΔIQ.Iv and CoreΔIQ.IIIv mutants, presenting the highest extent of attenuation, demonstrated decreased replication during infection in animals. However, CoreΔIQ.IIv and CoreΔIQ.IVv mutants exhibited more limited replication than parental BICv in cultured swine macrophages, but replicated almost as efficiently *in vivo* inducing disease similar to wild-type virus, although with a delayed progression. These two recombinant viruses likely do not contain sufficient mutations within the Core–IQGAP1 interaction to limit CSFV tropism *in vivo*, with viruses retaining the ability to replicate in multiple tissues and cause disease.

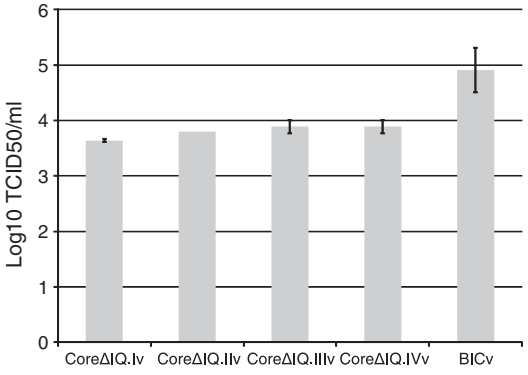

Fig. 3. *In vitro* growth characteristics of CoreΔIQv mutants and parental BICv. Primary swine macrophage cell cultures were infected (MOI = 0.01) with each of the mutants or BICv and virus yield titrated at 72 h post-infection in SK6 cells. Data represent means and standard deviations from two independent experiments. Sensitivity of virus detection: ≥log10 1.8 TCID50/ml.

The observed interaction of Core with the cell's cytoskeleton may inhibit *in vivo* host immune cell migration, hampering viral CSFV clearance. *Salmonella,* a facultative intracellular pathogen, releases a bacterial effector protein, SseI (also known as or SrfH), that is able to block the migration of host immune cells (McLaughlin et al., 2009). The mechanism involves the interaction of SseI with IQGAP1, reducing dendritic cell (DC) migration *in vivo* that in turn correlates with a reduction in the number of DC and CD4+ T cells in spleens of *Salmonella*-infected mice. *Salmonella* invasion promotes the interaction of IQGAP1 with Rho GTPases Rac1 and Cdc42 to induce actin polymerization (Brown et al., 2007). *In vitro*, knockdown of IQGAP1 significantly reduces *Salmonella* invasion and abrogates activation of Cdc42 and Rac1 by *Salmonella*. Similarly, Ibe, an effector protein of enteropathogenic and enterohemorrhagic *Escherichia coli* interacts with IQGAP1 (Buss et al., 2009). This factor co-localizes with IQGAP1 in *E. coli*-induced pedestals and actin-rich membrane ruffles. Interestingly, these pathogens use common effector mechanisms to increase infectivity. Experiments presented here demonstrated the existence of alterations in the distribution of the cytoskeleton of cells infected with CSFV. Actually, those alterations appear to be mediated by Core protein since mutations introduced in CoreΔIQ.Iv and CoreΔIQ.IIIv appear to disrupt the induction of those cytoskeleton alterations (Fig. 5).

CSFV Core protein seems to play an important role in virus virulence. In a previous study (Gladue et al., 2010) we observed that Core interacts with the cellular SUMOylation pathway proteins, SUMO-1 (small ubiquitin-like modifier) and UBC9, a SUMO-1 conjugating enzyme. In that study, specific residues in Core responsible for binding were shown important for CSFV pathogenesis since mutant viruses harboring substitutions at those positions were attenuated in swine. Interactions of viral proteins with the SUMOylation pathway seems to be important for viral infectivity, as shown for Ebola Virus Zaire VP35, Adenovirus CELO Gam1, Dengue Virus envelope protein, Human Herpesvirus 6 IE2, and Human Cytomegalovirus IE2, either preventing or inducing SUMO conjugation of target proteins (Ahn et al., 2001; Chang et al., 2009; Chiocca, 2007; Tomoiu et al., 2006). Here we extended the analysis of host factors interacting with CSFV proteins, specifically identifying IQGAP1 as a swine host protein binding partner for the CSFV Core protein. We also observed that mutations in the binding site in Core protein can completely abrogate CSFV virulence, demonstrating that acquisition of attenuation correlates with loss of binding to IQGAP1. These studies are providing interesting insights into the pathogenesis of CSFV by elucidating what appear to be multiple roles for Core in the CSFV cycle. Identifying cellular pathways and the viral proteins involved in infectivity could be important for developing better countermeasures to control virus infection in swine.

Materials and methods

Viruses and cells

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were cultured in Dulbecco's Minimal Essential Media (DMEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort

Table 2
Swine survival and fever response following infection with CSFV IQGAP1 mutants and parental BICv.

Virus	No. of survivors/total no.	Mean time to death (days ± SD)	No. of days to onset (days ± SD)	Fever	
				Duration no. of days (days ± SD)	Max daily temperature (± SD)
CoreΔIQ.Iv	6/6	No	No	No	102.8 (0.6)
CoreΔIQ.IIv	0/7	12 (0.5)	5.9 (2.5)	6.2 (2.7)	106.2 (1.1)
CoreΔIQ.IIIv	5/5	No	4.5 (0.7)[a]	1 (0)	103.8 (0.7)
CoreΔIQ.IVv	1/5	14 (2.4)	4 (0)	6.8 (3.6)	105 (1.2)
BICv	0/6	8.5 (1.9)	3.5 (0.6)	5 (1.6)	106.2 (0.7)

[a] Only one animal had a transient raised body temperature.

Collins, CO). Virulent CSFV Brescia strain was propagated in SK6 cells and used for the construction of an infectious cDNA clone (pBIC) (Risatti et al., 2005a). Growth kinetics was assessed on primary swine macrophage cell cultures prepared as described (Zsak et al., 1996). Titration of CSFV from clinical samples was performed using SK6 cells in 96-well plates (Costar, Cambridge, MA). Viral infectivity was detected, after 4 days in culture, by an immunoperoxidase assay using the CSFV monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$/ml. As performed, test sensitivity was ≥1.8 $TCID_{50}$/ml.

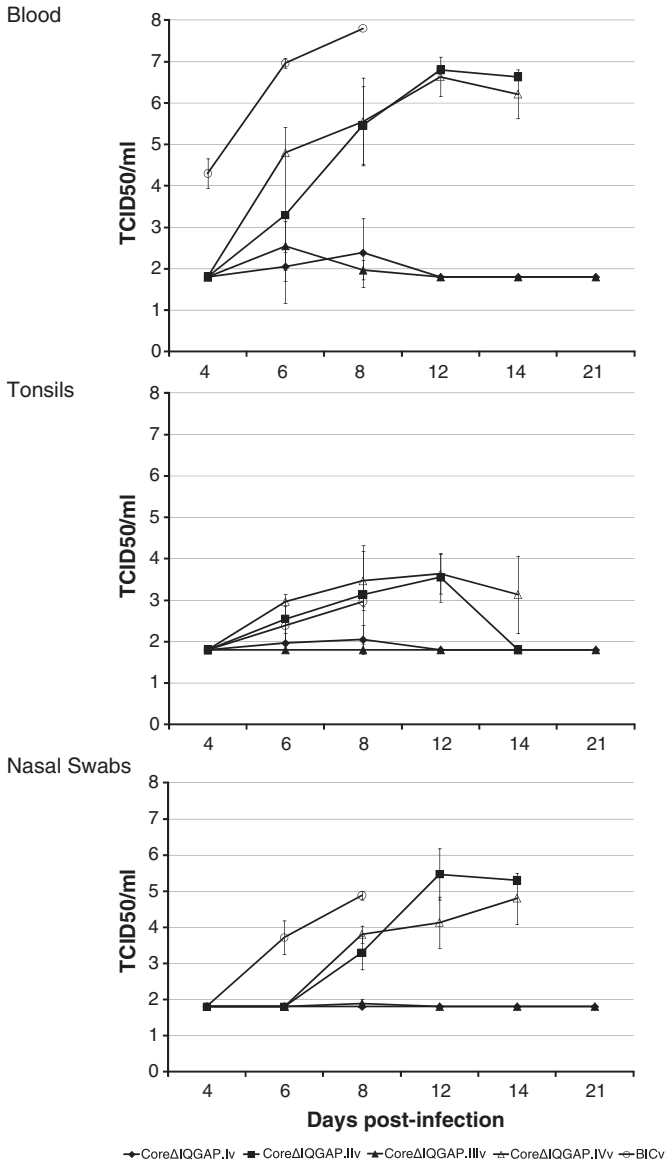

Fig. 4. Virus titers in clinical samples (blood, tonsil scrapings, and nasal swabs) from pigs infected with CoreΔIQ mutants and parental BICv. Each point represents the mean log10 TCID50/ml and standard deviations from at least two animals. Sensitivity of virus detection: ≥log 10 1.8 TCID50/ml.

Construction of CSFV CoreΔIQGAP (CΔIQ) mutants

Full-length pBIC was used as a template in which putative IQGAP1 binding sites in the Core protein were mutated. IQGAP1 binding sites were predicted using the Clustal W Analysis Program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using regions of the MMLV M protein that bind IQGAP1 as a template (Leung et al., 2006). Amino acids in the predicted binding regions were substituted with alanine, introduced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Cedar Creek, TX) performed per manufacturer's instructions. Primers were designed using the Stratagene Primer Mutagenesis program.

In vitro rescue of CSFV Brescia and CΔIQ mutants

Full-length genomic clones were linearized with *SrfI* and *in vitro* transcribed using the T7 MEGAscript system (Ambion, Austin, TX). RNA was precipitated with lithium chloride and transfected into SK6 cells by electroporation at 500 V, 720 Ω, 100 W with a BTX 630 electroporator (BTX, San Diego, CA). Cells were seeded in 12-well plates (Costar, Cambridge, MA) and incubated for 4 days at 37 °C and 5% CO_2. Virus was detected by immunoperoxidase staining as described above, and stocks of rescued viruses were stored at −70 °C.

DNA sequencing and analysis

Full-length clones and *in vitro* rescued viruses were completely sequenced with CSFV-specific primers by the dideoxynucleotide chain-termination method (Sanger et al., 1977). Sequencing reactions were prepared with the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Reaction products were sequenced on an ABI PRISM 3730xl automated DNA sequencer (Applied Biosystems, Foster City, CA). The final DNA consensus sequence represented an average five-fold redundancy at each base position. Sequence comparisons were conducted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Development of the cDNA library

A porcine primary macrophage cDNA expression library was constructed (Clontech, Mountain View, CA) using monocytes/macrophages obtained from healthy CSFV-free swine exactly as previously described (Gladue et al., 2010). Macrophage cultures were prepared from defibrinated swine blood. Total RNA were extracted from adherent cells using an RNeasy Mini kit (Qiagen, Valencia, CA). Contaminant genomic DNA was removed by DNase treatment using TURBO DNA-*free* (Ambion, Austin, TX). After DNase treatment, genomic DNA contamination of RNA stocks was assessed by real-time PCR amplification targeting the porcine β-actin gene. RNA quality was assessed using RNA

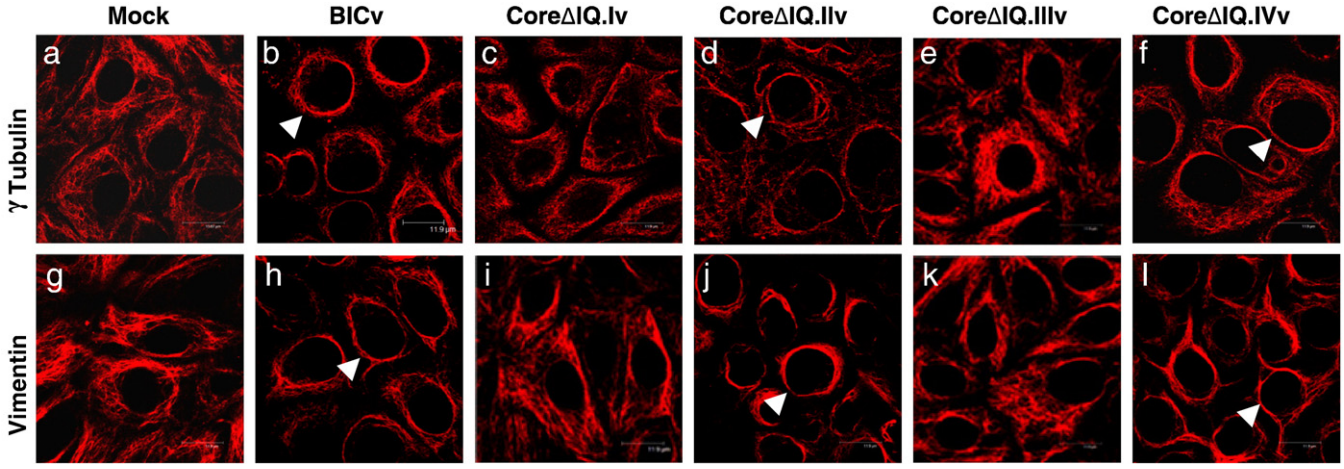

Fig. 5. Effect of BICv and CoreΔIQ viruses infection on host cell microtubules and intermediate filaments. Monolayers of SK6 cells were infected with BIC and CoreΔIQ viruses or mock infected, fixed with paraformaldehyde at 48 h post-infection, and processed for IF staining and confocal microscopy as described in Materials and methods. The microtubule was immunolabeled with mouse anti-γ-tubulin (a–f), and intermediate filaments with mouse-anti-vimentin (g–l). AlexaFluor 594-conjugate antibodies were used as secondary antibodies (red).

Nano Chips on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Cellular proteins were expressed as GAL4-AD fusion proteins while CSFV Core was expressed as GAL4-BD fusion proteins.

Library screening

The GAL4-based yeast two-hybrid system was used for this study (Chien et al., 1991; Fields and Song, 1989). The 'bait' protein, CSFV Brescia Core protein (amino acid residues 168–268 of the CSFV polyprotein), was expressed with an N-terminus fusion to the GAL4 Binding Domain (BD). As 'prey', the previously described swine macrophage cDNA library containing proteins fused to the GAL4 Activation Domain (AD) was used. Screening was done as previously described (Gladue et al., 2010). The IQGAP1 recovered from the library contained amino acids (502–704) of *homo sapiens* IQGAP1 (NCBI Reference Sequence: AAI39732) amino terminal fused to the GAL4-AD.

Infection of cells and confocal microscopy

Sub-confluent monolayers of SK6 cells grown on 12 mm glass coverslips in 24-well tissue culture dishes, were infected with BICv, CoreΔIQ.Iv, CoreΔIQ.IIv, CoreΔIQ.IIIv, CoreΔIQ.IVv at a multiplicity of infection (MOI) of 1–5 TCID50/cell, or mock infected for 48 h in Dulbecco's minimum essential medium (DMEM, Invitrogen, CA) containing 1% heat inactivated fetal bovine serum and 1% antibiotics. At forty-eight hours after infection the cells were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) and analyzed by confocal microscopy. Immunofluorescence and confocal microscopy using the antibodies listed below were performed as previously described (O'Donnell et al., 2005). Monoclonal antibodies against γ-tubulin (Sigma) and vimentin (Sigma), at a 1/100 dilution, were used as markers to identify microtubules and intermediate filaments respectively.

Animal infections

Each of the IQGAPv mutants was initially screened for their virulence phenotype in swine relative to the virulent Brescia strain. Swine used in these studies were 10 to 12 weeks old, forty-pound commercial breed pigs inoculated intranasally with 10^5 $TCID_{50}$ of either mutant or wild-type parental virus (BICv). Clinical signs (anorexia, depression, purple skin discoloration, staggering gait, diarrhea and cough) and changes in body temperature were recorded daily throughout the 21-day experiment. Total and differential white blood cell and platelet counts were obtained using a Beckman Coulter ACT (Beckman, Coulter, CA).

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